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PRINCIPAL INVESTIGATOR: Michael J. Imperiale, Ph.D.

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, Michigan 48109-1274

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Michael J. Imperiale, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
The University of Michigan
Ann Arbor, Michigan 48109-1274

*E-Mail:* imperial@umich.edu

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**13. ABSTRACT (Maximum 200 Words)**

BKV is a human polyomavirus that establishes a lifelong, persistent infection of the urinary tract. The virus encodes oncoproteins that induce tumors in animal models, and BKV DNA has been detected in human urinary tract tumors, including prostate. Prostate tumors have a relatively low frequency of mutations in the p53 and Rb1 genes, indicating that an agent such as a virus may be inactivating their functions. The aims of this proposal are to determine if BKV is present in prostate tumors and, if so, whether viral oncogenes are expressed. To accomplish this, normal and tumor tissue form individual patients will be analyzed. PCR, in situ PCR, and in situ hybridization will be performed to determine the presence of viral sequences, and RT-PCR and immunohistochemistry will be used to examine gene expression. Viral sequences from patients will be cloned and their function compared to wild type virus. This past year, we optimized most of these assays and began to analyze samples. We detect the presence and expression of the virus in a subset of normal and cancer cells. At this time, firm conclusions cannot be drawn as more samples need to be analyzed. If BKV is associated with some prostate cancers, our knowledge of the virus will be useful in designing drugs and vaccines for treatment.

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## Table of Contents

## INTRODUCTION

BK virus (BKV) is a human polyomavirus that establishes a lifelong, persistent infection of the urinary tract. The virus encodes two oncoproteins, the large T and small t antigens, that have been shown to cause cancer in animal models, and recently BKV DNA has been detected in various urinary tract tumors including tumors of the prostate. Among the spectrum of human cancers, prostate tumors have a relatively low frequency of mutations in the *p53* and *RB1* genes, indicating that an outside agent such as a virus may be inactivating the function of their gene products. Both large T and small t antigens are capable of doing so. The aims of this proposal are to determine if BKV is indeed present in these tumors and, if so, whether these oncogenic proteins are expressed and function to deregulate cell growth control.

## BODY

Most of our work during the second year of this project has been focused on optimizing the techniques required for these analyses. We then began the actual analysis of tumor samples. Our progress is described below after the appropriate items from the approved Statement of Work

Task 1. To examine prostate tumors and matched normal tissue for the presence of BKV sequences (months 1-30)

- isolate DNA and RNA from microdissected normal and tumor tissue (months 1-6)
- perform PCR analysis of DNA (months 3-18)

Last year at this time, we were having trouble with false positives in our solution PCR assays. We have solved this problem by designing new primer pairs that amplify larger segments of the viral genome, and by taking strict measures to avoid any possible contamination of samples and reagents. Figure 1 shows the specificity and sensitivity of the assay. We used BKV-specific primers to amplify plasmids containing either the BKV, JCV, or SV40 genome. The products were analyzed by agarose gel electrophoresis (top panel). We obtain a product using as little as 1 pg input BKV DNA, and based on the intensity of the product we believe we can detect even less. So signal is obtained even when using 10 ng of either of the other viruses. The specificity of the reaction is demonstrated in the bottom panel, where this gel was blotted and probed with our BKV-specific probe (same probe to be used for in situ hybridization, see below). Only the BKV products are recognized by the probe.

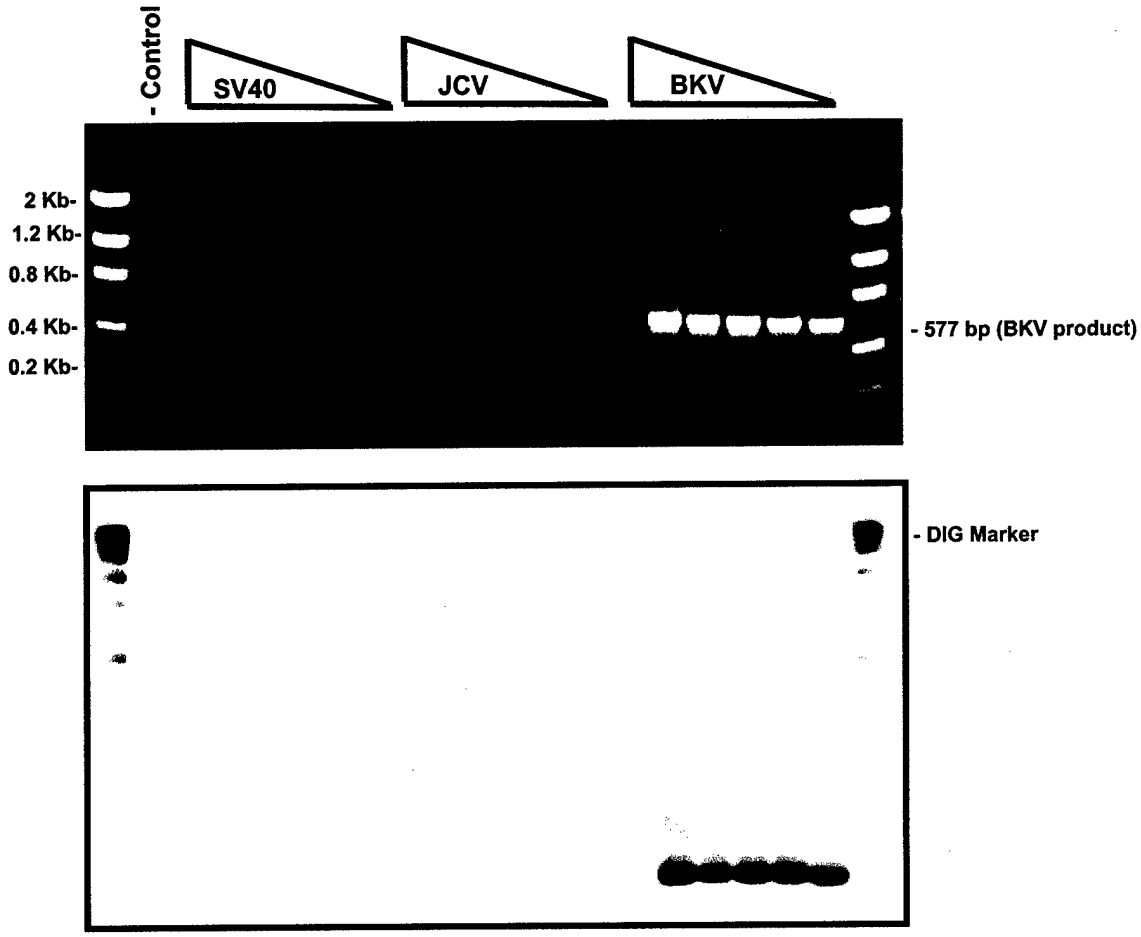
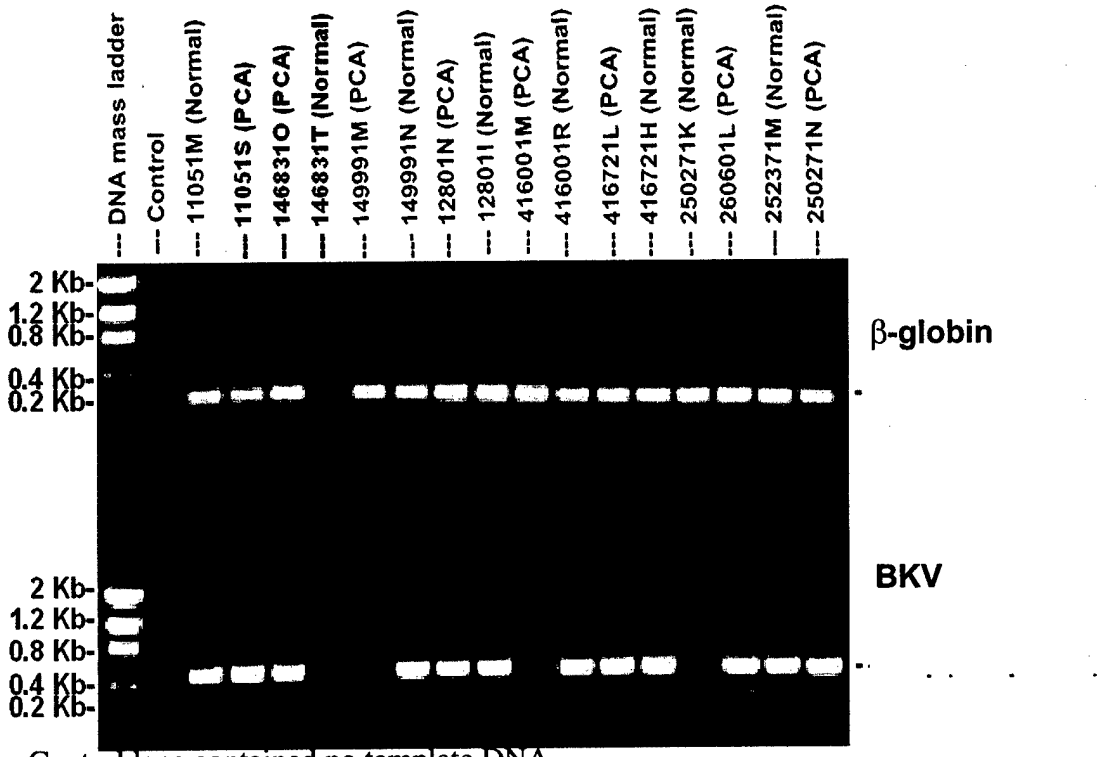



Figure 1. Assay parameters for amplification and detection of the BKV early region. DNAs were amplified using primers specific for the BKV early region. The amount of input DNA for each sample is, from left to right, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg. Top panel, EtBr-stained gel; Bottom panel, Southern blot using BKV-specific probe

Using these primers, we have assayed DNA extracted from approximately 15 patient samples to date. We have repeated each assay multiple times to ensure specificity and selectivity of the assay. We find viral DNA in both normal and cancer cells, in some of the samples. These results are shown in Figure 2, which is an EtBr-stained gel of the products using the same primers as in Figure 1 to amplify the BKV early region, or using primers specific for the β-globin gene to control for the integrity of the DNA templates. As discussed below, the sample size is too small at this point for us to reach any firm conclusions.




Control lane contained no template DNA.

- perform IS-PCR and ISH analysis of DNA (months 3-24)

We have developed conditions for in situ PCR as well as in situ hybridization. The in situ PCR assay was a little tricky to optimize as some patient samples are prone to incorporation of label in the absence of primers. We have solved this problem by pre-treating the samples with dideoxy nucleotide prior to the amplification: this prevents incorporation of label into nicked DNA, assuring that label will only be incorporated during extension of the primers. Using both these assays, once again we are finding viral DNA in normal cells as well as areas of abnormal histology, but not in all samples. Examples of positive ISH results are shown in Figure 3. We detect viral DNA using the specific probe but not a scrambled oligonucleotide control, and only in prostate samples, not in breast.

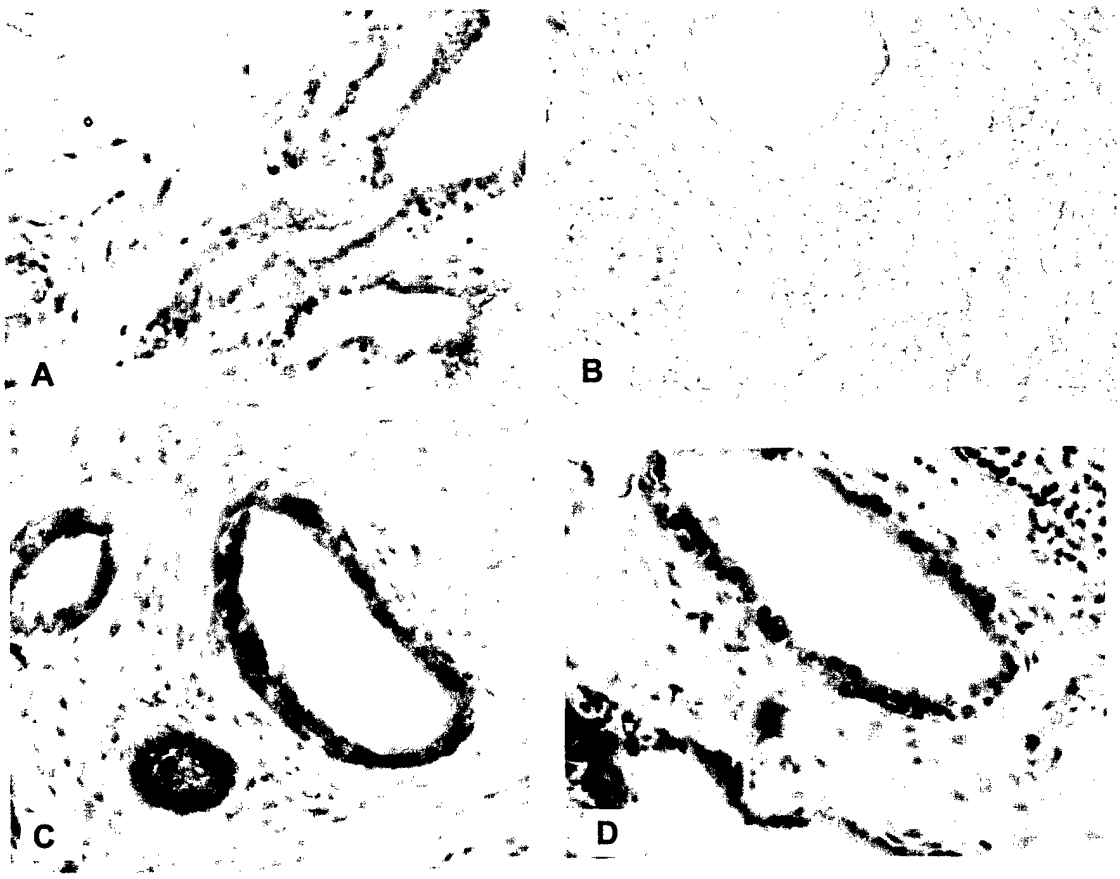



Figure 3. In situ hybridization analysis of prostate sections. A, prostate, control probe (scrambled oligonucleotide of same length and G+C content); B, breast, BKV probe; C, D, prostate, BKV probe.

- perform sequence analysis of DNA (months 6-24)
- perform RT-PCR and IS-RT-PCR analysis of RNA (months 12-30)

We have sequenced a very small number of the products from the solution PCR analysis. This analysis has confirmed that we are amplifying the BKV early region (Figure 4). The results show that the products are indeed BKV and not JCV or SV40. We have not detected any polymorphisms but, again, the sample size is small.

```
6721L       ---TTTNGGCCAGATACCCTGTACTGCAAGGAATGGCCTATTTGTTCCAAAAAGCCTTCT
6721H       ----TTTGSCCAGATACCCTGTACTGCAAGGAATGGCCTATTTGTTCCAAAAAGCCTTCT
BKV         CCTCTTTGCCCAGATACCCTGTACTGCAAGGAATGGCCTATTTGTTCCAAAAAGCCTTCT
JCV         CCTCCTAATTCTGATACCCTTTATTGCAAGGAATGGCCTAACTGTGCCACTAATCCTTCA
SV40        AATCCTGGTGTTGATGCAATGTACTGCAAACAATGGCCTGAGTGTGCAAAGAAAATGTCT

6721L       GTGCACTGCCCTTGCATGCTATGTCAGCTTAGATTAAGGCATTTAAATAGAAAATTTTTA
6721H       GTGCACTGCCCTTGCATGCTATGTCAGCTTAGATTAAGGCATTTAAATAGAAAATTTTTA
BKV         GTGCACTGCCCTTGCATGCTATGTCAGCTTAGATTAAGGCATTTAAATAGAAAATTTTTA
JCV         GTGCATTGCCCCTGTTTAATGTGCATGCTAAAATTAAGGCATAGAAACAGAAAATTTTTA
SV40        GCTAACTGCATATGCTTGCTGTGCTTACTGAGGATGAAGCATGAAAATAGAAAATTATAC

6721L       AGAAAAGAGCCCT-TGGTTTGGATAGATTGCTACTGCATTGACTGCTTCACACAGTGGTT
6721H       AGAAAAGAGCCCT-TGGTTTGGATAGATTGCTACTGCATTGACTGCTTCACACAGTGGTT
BKV         AGAAAAGAGCCCT-TGGTTTGGATAGATTGCTACTGCATTGACTGCTTCACACAGTGGTT
JCV         AGAAGC-AGCCCACTTGTGTGGATAGATTGCTATTGCTTTGATTGCTTCAGACAATGGTT
SV40        AGGAAAGATCC-ACTTGTGTGGGTTGATTGCTACTGCTTCGATTGCTTTAGAATGTGGTT

6721L       TGGCTTAGACCTAACTGAAG-AAACT--CTGCAATGGTGGGTCCAAATAATTGGAGAAAC
6721H       TGGCTTAGACCTAACTGAAG-AAACT--CTGCAATGGTGGGTCCAAATAATTGGAGAAAC
BKV         TGGCTTAGACCTAACTGAAG-AAACT--CTGCAATGGTGGGTCCAAATAATTGGAGAAAC
JCV         TGGGTGTGACTTAACCCAAG-AAGCT--CTTCATTGCTGGGAGAAAGTTCTTGGAGACAC
SV40        TGGACTTGATCTTTGTGAAGGAACCTTACTTCTGTGGTGTG---ACATAATTGGACAAAC

6721L       TCCCTTCAGAGATCTAAAGCTTTAAGGTAACTAACT---TATATTT---AGATAAATAAT
6721H       TCCCTTCAGAGATCTAAAGCTTTAAGGTAACTAACT---TATATTT---AGATAAATAAT
BKV         TCCCTTCAGAGATCTAAAGCTTTAAGGTAACTAACT---TATATTT---AGATAAATAAT
JCV         CCCCTACAGGGATCTAAAGCTTTAAGGTAAACCACTATTTTTCTTTTGCAGGGCATT--T
SV40        TACCTACAGAGATTTAAAGCTCTAAGGTAAATA--TA---AAATTTTTAAGTGTA----T

6721L       AAAATATTAAAAGGCCCTAAG--TAATTATTTTT---TTTATAGGTGCCAACCTATGGAA
6721H       AAAATATTAAAAGGCCCTAAG--TAATTATTTTT---TTTATAGGTGCCAACCTATGGAA
BKV         AAAATATTAAAAGGCCCTAAG--TAATTATTTTT---TTTATAGGTGCCAACCTATGGAA
JCV         TGTTTTTTACAATAAACT-----TAATTGTTTTT---TTT-TAGGTGCCAACCTATGGAA
SV40        AATGTGTTAAACT--ACTGATTCTAATTGTTTGTGTATTT-TAGATTCCAACCTATGGAA

6721L       CAGAAGAGTGGGAGTCCTGGTGGAGTTCCTTTAATGAAAAATGGGATGAAGATTTATTTT
6721H       CAGAAGAGTGGGAGTCCTGGTGGAGTTCCTTTAATGAAAAATGGGATGAAGATTTATTTT
BKV         CAGAAGAGTGGGAGTCCTGGTGGAGTTCCTTTAATGAAAAATGGGATGAAGATTTATTTT
JCV         CAGATGAATGGGAATCCTGGTGGAATACATTTAATGAGAAGTGGGATGAAGACCTGTTTT
SV40        CTGATGAATGGGAGCAGTGGTGGAATGCCTTTAATGAG----------GAAAACCTGTTTT

6721L       GCCATGAAGATATGTTTGCCAGTGATGAAGAAGCAACAGC
6721H       GCCATGAAGATATGTTTGCCAGTGATGAAGAAGCAACAGC
BKV         GCCATGAAGATATGTTTGCCAGTGATGAAGAAGCAACAGC
JCV         GCCATGAAGAAATGTTTGCCAGTGATGATGAA--AACACA
SV40        GCTCAGAAGAAATGCCATCTAGTGATGATGAGGC------
```

Figure 4. Sequence alignment of PCR products. The sequence of two of the PCR products (top two sequences) are aligned with BKV, JCV, and SV40.

As we discussed in last year's report, we have added immunohistochemical assays for T antigen to this task. All samples have been analyzed by this method and a fraction of the samples are positive for T antigen expression. Examples of positive IHC sections are shown in Figure 5. No signal is obtained in breast sections, or in prostate sections that have been probed with a control antibody.

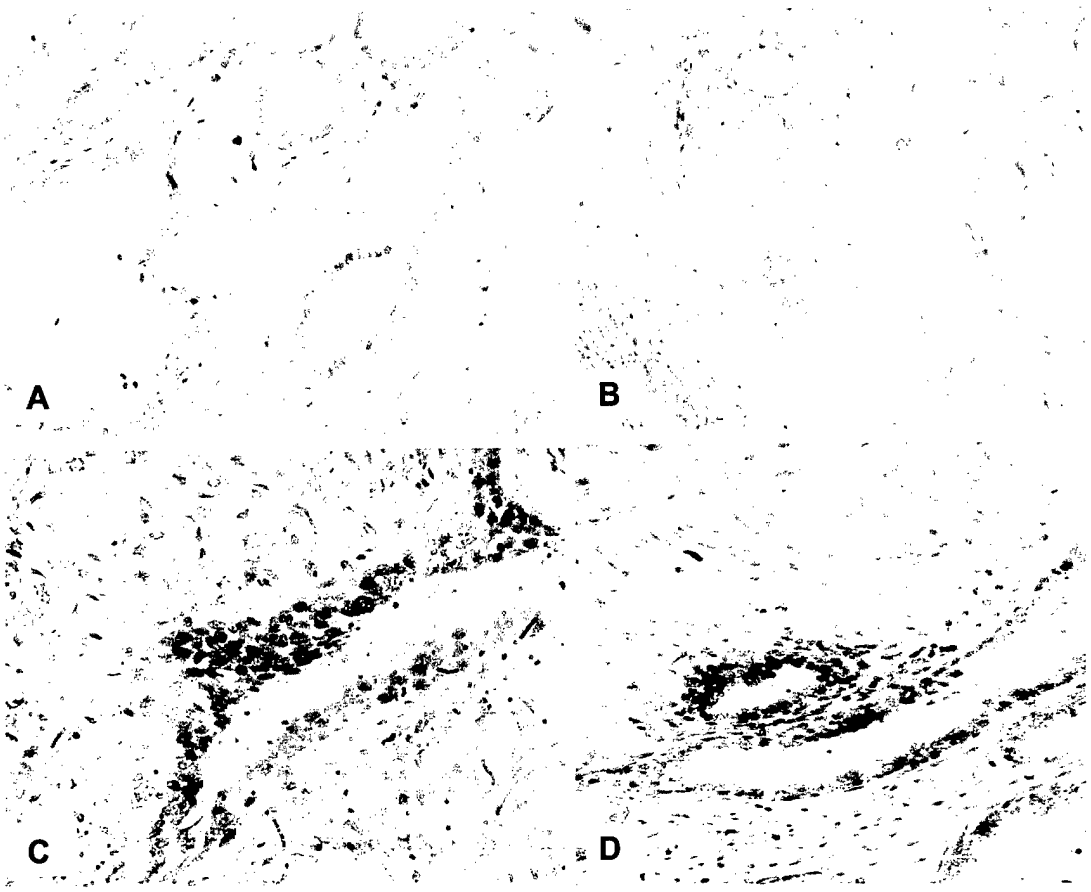



Figure 5. Immunohistochemical analysis of prostate samples. Sections were stained with a monoclonal antibody against BKV large T antigen, followed by alkaline phosphatase-conjugated secondary antibody. A, prostate, control antibody; B, breast, anti-T antigen; C, D, prostate, anti-T antigen.

The results from Task 1 to date are summarized in Table 1. Note that the table summarizes the results of multiple repeats of each assay.

**Table 1**
**Summary of Analysis of Prostate Samples for BKV**

| Sample | pathology[a] | PCR | ISH | IHC |
|---|---|---|---|---|
| 271N | Ca | + | + | + |
| 271K | N | - | + | + |
| 601 | Ca | + | + | - |
| 2371 | N | + | + | - |
| 1051M | N | + | - | + |
| 1051S | Ca | + | - | + |
| 6831O | Ca | + | + | - |
| 6831T | N | - | - | - |
| 9991M | Ca | - | + | - |
| 9991N | N | + | - | - |
| 2801N | Ca | + | + | - |
| 2801I | N | + | + | - |
| 6001M | Ca | - | + | - |
| 6001R | N | + | - | - |
| 6721L | Ca | + | + | + |
| 6721H | N | + | + | + |

[a]N = normal, Ca = abnormal

Task 2. To determine how alterations in viral sequences in tumors affect the replication and transformation properties of the virus (months 9-36)

- construct genomic viral clones containing patient-derived sequences (months 9-24)

We have not yet cloned out any patient derived sequences since, so far, they are identical to wild type virus. However, we have not performed PCR analysis using primers that amplify the regulatory region. We are currently beginning these studies, and at the present time are optimizing the primers and amplification conditions.

- determine the ability of these clones to replicate in permissive host cells (months 12-36)
- construct early region expression vectors containing patient-derived sequences (months 9-24)
- determine the ability of these clones to interfere with normal growth control pathways involving pRb and p53 (months 18-36)

KEY RESEARCH ACCOMPLISHMENTS

- development of solution PCR assay for BKV sequences in microdissected tissue

- development and optimization of immunohistochemical staining procedures for BKV T antigen in human tissue
- detection of BKV T antigen in human prostate tissue
- development of in situ PCR assays
- development of in situ hybridization assays

## REPORTABLE OUTCOMES

Three abstracts were presented at international meetings this year:

1. Oral presentation by Dr. Imperiale:

Imperiale, M.J., Low, J.A., Das, D., and Robertson, E. (2003). BKV infection of humans and human cells. First International Symposium, "Polyomaviruses and Human Diseases: Basic and Clinical Perspectives," Florence, Italy.

2. Poster presentation by Dr. Das:

Das, D., Shah, R.B., and Imperiale, M.J. (2003). Analysis of prostate tissue for human polyomaviruses (BK and JC) and SV40. American Society for Virology 22$^{nd}$ Annual Meeting, Davis, CA.

3. Oral presentation by Dr. Imperiale:

Low, J.A., Szczypka, M., Das, D., Humes, H.D., and Imperiale, M.J. (2003). Interaction of BKV with the human urinary tract. ICGEB DNA Tumour Virus Meeting, Trieste, Italy.

## CONCLUSIONS

Since last year, we have optimized all of the DNA detection assays and begun to analyze patient samples. We have decided that each sample must be assayed multiple times, with as many of the approaches as is feasible, in order to convince ourselves of whether a sample is positive or negative. We are finding viral DNA in a subset of the samples, in both normal and abnormal cells. At this time, the sample size is too small to allow any firm conclusions. There is one trend, however, which is that we have not seen any samples in which normal cells are positive and abnormal cells are not. The finding of the virus in normal cells has complicated the analysis, however. We are currently working with a biostatistician to determine the best methods that will allow us to draw concrete conclusions about whether the presence of the virus in neoplastic samples in significant or not. During the coming year we will be able to assay many more samples, now that the conditions are set.

REFERENCES

none

APPENDICES

copies of abstracts reported above in the Outcomes section

# Analysis of Prostate Tissue for Human Polyomaviruses (BK and JC) and SV40

[1]Dweepanita Das, [2]Rajal B. Shah and [1, 3, 4]Michael J. Imperiale
[1]Department of Microbiology and Immunology, [2]Department of Pathology and Urology, [3]Comprehensive Cancer Center, and [4]Center for Gene Therapy
University of Michigan Medical School, Ann Arbor, Michigan 48109-0942

Prostate cancer is a common malignancy in older American men. Technological advances in the field of Polymerase Chain Reaction (PCR) have made it possible to localize DNA/RNA in tissues with non-radioactive labels. BKV is a human polyomavirus that establishes a lifelong, sub clinical persistent infection of the urinary tract in immunocompetent individuals. When the immune system is compromised, however, it can cause severe disease in the kidney and bladder. BKV sequences have also been detected in urinary tract neoplasms, prompting investigators to propose that this virus may contribute to cancer through the action of its two early proteins, large T and small t antigens. We are therefore interested in analyzing prostate tumors not only for BKV but also for related virus, JCV and SV40, and if present to better characterize these sequences. Hence, utilizing PCR, we are analyzing normal and tumor prostate tissues for human polyomavirus sequences (BKV and JCV) and SV40. We have successfully optimized the following techniques: in situ PCR, PCR in situ hybridization and Immunohistochemistry. Primers used in our studies are to the early region of BKV, JCV or SV40 and each primer set is very virus specific. This will aid in differentiating between BKV, JCV and SV40. Our preliminary study with matched pairs (paraffin embedded) of normal and tumor tissue sections shows the presence of BKV sequences in these tissues. Additionally, our preliminary data from immunohistochemical analysis using a monoclonal antibody specific to T antigen shows the presence of T antigen in some of the samples tested. We are currently in the process of screening fresh frozen prostate tissues for these viral sequences by PCR followed by Southern blot. This will be followed by real time RT-PCR and sequence analysis. These extensive studies will help determine whether polyomavirus is important in prostate carcinogenesis.

# BKV Infection of Humans and Human Cells

Michael J. Imperiale, Jonathan Low, Dweepanita Das, and Erin Robertson, Department of Microbiology and Immunology and Comprehensive Cancer Center, University of Michigan Medical School, 1500 E Medical Center Dr, Ann Arbor, MI 48109 USA

Until fairly recently, the human polyomavirus BKV was largely ignored because it did not appear to be a major cause of disease. Data are accumulating, however, that BKV infections are a serious problem in renal transplant patients, and BKV sequences have been detected in a variety of human tumors. In order to understand the molecular biology of BKV in more detail, we undertook an analysis of the major transforming protein, large T antigen (TAg). We demonstrated that TAg induces serum-independent growth in monkey kidney cells by disrupting the pRb tumor suppressor pathway. An interesting observation we made during the course of those experiments, however, was that it was very difficult to isolate cells lines stably expressing TAg. The question arises, then, of how the molecule can have two apparently disparate functions, one inhibitory to cell growth and the other growth-promoting. In order to address this issue and to understand in more detail the interactions between the virus and the infected cell, we have begun to analyze the behavior of BKV in what is likely its normal host cell, primary kidney proximal tubule epithelial cells. BKV infects and replicates in these cells quite efficiently, and we are currently analyzing its effects on cell gene expression and growth. These data have important implications for how the virus establishes a persistent infection and how it might be reactivated in immunocompromised individuals. We have also begun to examine prostate tissue, both normal and diseased, for the presence of BKV. Our preliminary data indicate that the virus is present in both sets of samples, and we are currently assessing whether there are differences in viral gene expression that might correlate with oncogenesis.

**ICGEB DNA TUMOUR VIRUS MEETING**
**15-20 July 2003**

To be submitted as an e-mail attachment to:
Ms. E. Lippolis
ICGEB, Trieste, Italy
E-mail: lippolis@icgeb.org

ABSTRACT SUBMISSION FORM
(Closing date for submission: 15 April 2003)


Presenting Author: Michael Imperiale
Address: University of Michigan Medical School
E-mail: imperial@umich.edu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[ ] poster

**please indicate session:**
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[ ] Gene Expression
[ ] Replication
[ X ] Viral Pathogenesis
[ ] Cell Growth / Death
[ ] Virus Host Interactions
[ ] Virions / Vectorology / Gene Therapy

**Format:**

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- Do not use ALL CAPS, except for scientific definitions
- Font Palatino size 10
- Single-line spacing, justified, no line between paragraphs

**Interactions of BKV with the Human Urinary Tract**


*Jonathan A. Low[1], Mark Szczypka[2], Dweepanita Das[1], H. David Humes[3], and Michael J. Imperiale[1]*
Departments of Microbiology and Immunology[1] and Internal Medicine[3], University of Michigan Medical School, and Nephros Therapeutics[2], Ann Arbor, MI 48109 USA



BK virus (BKV), a member of the polyomavirus family, has been found to be nearly ubiquitous in the human population in the form of persistent, sub-clinical infections. Although quite a bit is known about how polyomaviruses interact with established cell lines, little is known about the exact nature of the interaction of BKV with its natural host cell from human urinary tract epithelium. We obtained human proximal tubule kidney epithelial cells and cultured them under conditions in which they are capable of maintaining their differentiated structure and function. We performed in vitro infections of these proximal tubule cells with BKV. We isolated both protein and episomal DNA from these infected cells and determined the growth and expression patterns of BKV. We are particularly interested in the expression of BKV large T antigen and the effects it may have on the infected kidney epithelial cells. In addition, we have examined SV40 infection of these cells, and while BKV replicates well, SV40 does not appear to productively infect them.

BKV sequences have been reported to be present in urinary tract neoplasms, prompting investigators to propose that this virus may contribute to cancer through the action of its two early proteins, large T and small t antigens. We have been analyzing human prostate tissue for the presence and expression of BKV sequences. Our preliminary studies on paraffin embedded normal and tumor tissue sections, utilizing in situ PCR and in situ hybridization, show the presence of BKV

sequences within these prostate tissues. Additionally, our preliminary immunohistochemical analysis using a monoclonal antibody specific to T antigen shows the presence of T antigen in some of the samples tested. We are currently in the process of screening a larger number of samples and are also attempting solution PCR analyses from paraffin embedded and fresh frozen prostate tissues in order to perform sequence analysis and functional studies. These studies will help indicate whether BKV plays a role in prostate carcinogenesis.